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CYCLE XXIX

HEPCIDIN SUPPRESSION IN BETA-THALASSEMIA CARRIERS WITH IRON OVERLOAD

Preliminary reports from an ongoing study

S.S.D. MED 09

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LIST OF ABBREVIATIONS

βTT	β-thalassemia trait
DFO	Deferoxamine
DIOS	Dysmetabolic iron overload syndrome
EPO	Erythropoietin
ERFE	Erythroferrone
IO	Iron overload
ESAs	Erytropoietic-stimulating agents
FPN	Ferroportin
GDF15	Growth differentiation factor 15
HH	Hereditary Hemochromatosis
HIF1-α	Hypoxia inducible factor-1
LIC	Liver iron concentration
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NGS	Next Generation Sequencing
NTBI	Non-transferrin-bound iron
NTDT	Non-transfusion-dependent thalassemias
RBC	Red blood cells
ROS	Reactive oxygen species
sTfR	Soluble transferrin receptor
TI	Thalassemia intermedia
TS	Transferrin saturation

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ABSTRACT

A certain degree of iron overload (IO) is sometimes seen in subjects with β-thalassemia trait (βTT), a mild form of non-transfusion-dependent β-thalassemia.

The pathogenesis is unclear, but recently a population study in Sri Lankan children has showed that βTT is characterized by mild hepcidin suppression due to increased erythropoietic activity. This data is in accordance with previous other studies suggesting that erythropoietic drive can regulate hepcidin production, potentially acting via an erythroid-derived hormone, such as the recently identified erythroferrone (ERFE). Hepcidin is the master regulator of iron homeostasis which acts by inhibiting dietary iron absorption and iron release from macrophages, thus its suppression leads to an increased of iron availability in the body. In βTT patients, hepcidin defect may be further aggravated by genetic (i.e. mutations in hemochromatosis genes) or acquired factors (e.g. alcohol abuse or non-alcoholic liver diseases).

Treatment of IO in βTT is problematic, since "standard" large-volume phlebotomies are not feasible in mildly anaemic subjects, as well as the lack of approval of oral iron chelators and of specific guidelines. Deferoxamine is the only approved therapy, but it is poorly applicable because of parenteral administration and side effects. Sporadic case reports have suggested the use of phlebotomies in βTT patients with IO, but feasibility and efficacy of such approach has not been evaluated in patients' series.

This study has been designed to better characterize factors involved in the development of IO in βTT patients, and to evaluate feasibility and efficacy of "mini-phlebotomies" in this condition.

In our population, a substantial alcohol consumption (>100 g/day) appears a common acquired cofactor in βTT patients with IO, while biomolecular analysis did not reveal potentially pathogenic variants out of the known H63D and C282Y in HFE. Mean hepcidin levels were higher than those observed in the general population, suggesting that the hepcidin response to IO is conserved, while the

hepcidin:ferritin ratio was lower than in age- and sex-matched normal individuals, in agreement with a relative hepcidin defect in βTT.

Fifteen patients started treatment with "mini-phlebotomies", eleven of them have reached the iron depletion and no one experienced a worsening of anaemia during the treatment, suggesting mini-phlebotomies as a valuable approach for this peculiar category of patients.

1. INTRODUCTION

1.1 Beta-thalassemia syndromes

Beta-thalassemias are a group of hereditary blood disorders characterized by abnormalities in the synthesis of the beta globin chains of hemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals [Galanello R et al, 2010]. Three main forms have been described: thalassemia major (also called "Cooley's disease" or "Mediterranean Anemia"), thalassemia intermedia, and thalassemia minor (variably referred to as "beta-thalassemia carrier" or "beta-thalassemia trait").

Individuals with thalassemia major usually present within the first two years of life with severe anemia, requiring regular red blood cells (RBC) transfusions.

Thalassemia minor is clinically asymptomatic, with the majority of subjects showing only a mild hypochromic microcytic anemia.

In the middle lies thalassemia intermedia (TI), a condition too mild to be considered thalassemia major and too severe to be called thalassemia minor. Individuals with TI present later than thalassemia major, have milder anemia (with hemoglobin levels ranging between 7 and 10 g/dl), and by definition do not require or only occasionally require transfusions. Sometimes, they are completely asymptomatic until adult life. While in the past the TI diagnosis was based only on approximate clinical and biochemical criteria (such as hemoglobin levels), over the past decade, our understanding of thalassemia intermedia has increased enormously thanks to advances in molecular investigations [Haddad A et al, 2014]. This aspect is very important, because it is now clear that TI presents pathophysiological mechanisms, clinical presentation as well as complications associated with the disease that are different from those of β-thalassemia major and minor.

Thalassemia is among the most common genetic disorders worldwide. It has been estimated that about 1.5% of the global population (80 to 90 million people) are carriers of beta-thalassemia, with about 60,000 symptomatic individuals born annually, the great majority in the developing world [Origa R, 2016].

Beta-thalassemia is prevalent in Mediterranean countries (including Italy), the Middle East, Central Asia, India, Southern China, and the Far East, as well as countries along the north coast of Africa and in South America. The highest carrier frequency is reported in two of the largest Mediterranean islands: Cyprus (14%) and Sardinia (10.3%), and in Southeast Asia [Flint J et al, 1998]. The high frequency of beta-thalassemia alleles in these regions is most likely related to the selective pressure from *Plasmodium falciparum* malaria. Due to the frequency of immigration from these regions in recent decades, the incidence of the thalassemias has increased also in other geographical areas.

Beta-thalassemias are caused by point mutations (more than 200 have been so far reported) or, more rarely, deletions in the beta globin gene on chromosome 11, leading to reduced ($beta^{+}$) or absent ($beta^{0}$) synthesis of the beta chains of hemoglobin [Giardine B et al, 2007]. Transmission is typically autosomal recessive. A list of common mutations in Mediterranean area according to the severity of defect is reported in Table 1.

Table 1: Common types of beta-thalassemia mutations in Mediterranean region.

β-gene mutation	Severity
-101C>T	β^{++}
-87C>G	β^{++}
IVS1-nt1G>A	β^{0}
IVS1-nt6T>C	$\beta^{+/++}$
IVS1-nt110G>A	β^{+}
IVS1-nt745C>G	β^{+}
Codon 39 C>T	β^{+}
Codon 5 –CT	β^{0}
Codon 6 –A	β^{0}
AATAAA to AATGAA	β^{++}
Codon 27 G>T Hb (Hb	β^{++}

[Modified from Galanello R et al, 2010]

β^{0}: complete absence of beta globin on the affected allele
β^{+}: residual production of beta globin (around 10%)
β^{++}: very mild reduction in beta globin production

Although the degree of globin chain reduction is determined by the nature of the mutation at the beta globin gene located on chromosome 11, several modifier genes contribute to differences in disease phenotype. In particular, because the main pathophysiological determinant of the severity of the β-thalassemia

syndromes is the extent of α/non-α-globin chain imbalance, any factor capable of reducing this imbalance results in a lesser degree of α-globin chain precipitation and may ameliorate the clinical picture.

Factors include the presence of silent or mild beta-thalassemia alleles associated with a high residual output of beta globin, the coinheritance of alpha thalassemia and/or of genetic determinants able to sustain a continuous production of gamma globin chains (HbF) in adult life [Galanello R et al, 1998].

In some instances, heterozygous beta-thalassemia may lead to the thalassemia intermedia phenotype instead of the asymptomatic carrier state. Most of these patients have excess functional alpha globin genes (alpha gene triplication or quadruplication) which increase the imbalance in the ratio of alpha/non-alpha globin chain synthesis [Sollaino MC et al, 2009; Origa R et al, 2014].

However, the clinical severity of β-thalassemia syndromes is also influenced by genetic factors unlinked to globin genes [Origa R, 2016] as well as environmental conditions and management.

Table 2 summarizes genetic basis and clinical manifestations of common β-thalassemia syndromes.

Table 2: Genetic basis and clinical manifestations of common β-thalassemias.

Features	Thalassemia Major	Thalassemia Intermedia	Thalassemia Minor
Genetic pathology	Two β-globin genes carrying a severe thalassemia mutation	Two β-globin genes carrying a thalassemia mutation, at least one of which is mild; one β-globin thalassemia mutation in combination with excess α-globin genes (less common)	One β-globin gene carrying a thalassemia mutation
Clinical manifestations	Severe anemia requiring regular transfusions beginning in infancy; splenomegaly and bone disease depending on efficacy of transfusion therapy; severe iron overload	Mild to moderate anemia; relative independence from transfusions; prominent splenomegaly and bone deformities; variable degrees of iron overload depending on severity of anemia and transfusion requirement	Mild or no anemia, with variable microcytosis; no splenomegaly; no bone disease; possible iron overload in presence of cofactors
Severity	Lifelong supportive care required	From asymptomatic to severely symptomatic	Asymptomatic

[Modified from Rund D et al, 2005]

Ineffective erythropoiesis and hemolysis together cause the anemia that occurs in thalassemia. The reduced amount or absence of beta globin chains result in a relative excess of unbound alpha globin chains that precipitate in erythroid precursors in the bone marrow, leading to their premature death and hence to ineffective erythropoiesis (the bone marrow of patients with thalassemia contains five to six times the number of erythroid precursors as does the bone marrow of healthy controls, with 15 times the number of apoptotic cells in the polychromatophilic and orthochromic stages). Peripheral hemolysis occurs when insoluble alpha globin chains induce membrane damage to the peripheral erythrocytes. Anemia stimulates the production of erythropoietin with consequent intensive but ineffective expansion of the bone marrow (up 25 to 30 times

normal), which in turn causes the typical described bone deformities. Prolonged and severe anemia and increased erythropoietic drive also result in hepatosplenomegaly and extramedullary erythropoiesis.

Figure 1 illustrates the complex chain of events that occurs in erythrocytes, resulting in their accelerated peripheral destruction.

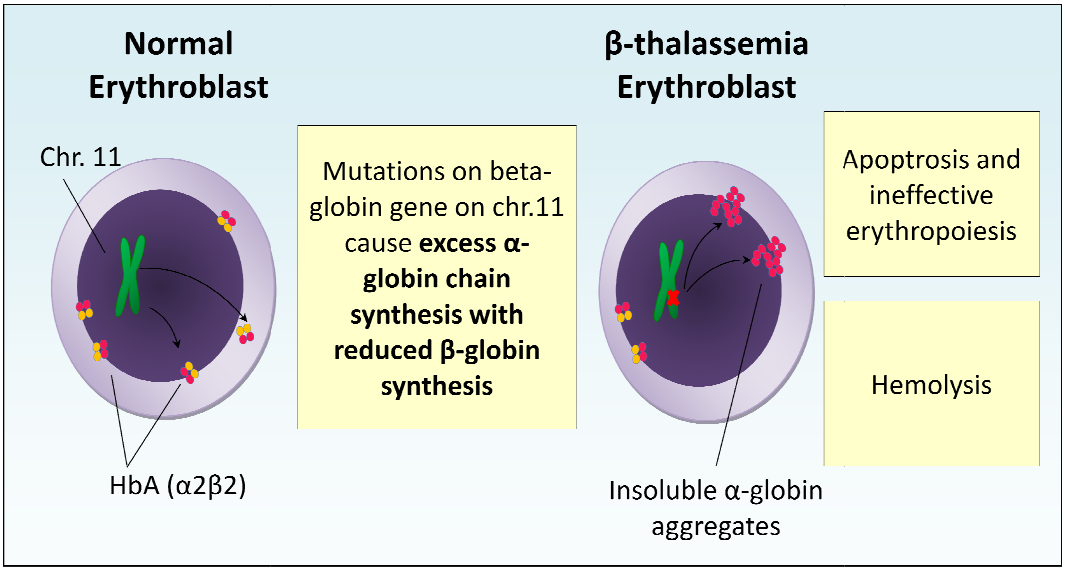

Figure 1: Pathophysiological mechanisms in beta-thalassemias.

The severity of β-thalassemia is related mainly to the degree of α-globin chain excess, which precipitates in the red blood cell precursors, causing both mechanic and oxidative damage (ineffective erythropoiesis and hemolysis).

1.2 Ineffective erythropoiesis and regulation of iron status

Iron is essential for heme and iron-sulfur cluster synthesis in every cell of the body but is required in larger amounts for hemoglobin synthesis during terminal erythropoiesis. Basal systemic iron homeostasis is maintained primarily by the recycling of iron from senescent erythrocytes (approximately 20-25 mg every day) with a small contribution from intestinal absorption (about 1-2 mg daily). During times of stress erythropoiesis, iron consumption by the bone marrow can increase up to 10-fold [Kim A et al, 2015; Camaschella C et al, 2016].

The primary regulator of systemic iron homeostasis, and thus iron availability for erythropoiesis, is hepcidin, a 25-amino-acid peptide hormone that is produced by hepatocytes [Park CH et al, 2001]. Hepcidin acts by binding to ferroportin, the

sole known cellular iron exporter, and causing its internalization and degradation within lysosomes [Nemeth E et al, 2004]. As ferroportin is expressed on duodenal enterocytes, macrophages and hepatocytes, hepcidin controls the flow of iron from gut absorption, recycling of senescent erythrocytes, and cellular iron stores (Figure 2). Injecting a single dose of synthetic hepcidin into mice caused a rapid and dramatic drop in serum iron. Prolonged hepcidin overexpression in transgenic mice resulted in iron-restricted erythropoiesis and iron deficiency. Conversely, inactivation of the hepcidin gene in mice caused severe iron overload, and mutations in the hepcidin gene in humans are associated with juvenile hemochromatosis, a particularly severe form of genetic iron overload [Ganz T, 2011; Ganz T et al, 2012].

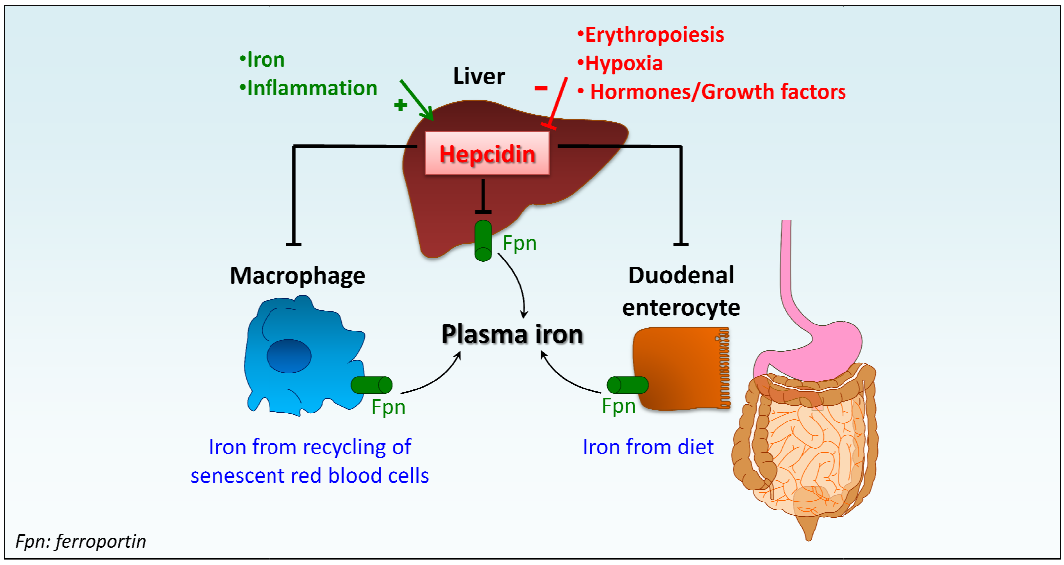

Figure 2: Hepcidin has a central role in maintenance of iron homeostasis.

Hepcidin synthesis is regulated by multiple stimuli. Intracellular and extracellular iron concentrations and inflammation stimulate hepcidin transcription, whereas increased erythropoietic activity suppresses hepcidin production. In turn, hepcidin regulates plasma iron concentrations by controlling ferroportin concentrations on iron exporting cells including duodenal enterocytes, recycling macrophages of the spleen and. liver, and hepatocytes.

In anemias with ineffective erythropoiesis such as β-thalassemia, hepcidin production is suppressed [Pasricha SR et al, 2013; Ramos P et al, 2010; Origa R et al, 2007], causing hyperabsorption of dietary iron by enterocytes and systemic iron overload even in the absence of transfusions. Although increased

erythropoietic activity has long been known to suppress hepcidin expression, the specific mechanisms and "erythroid regulators" involved are still under investigation. Multiple proteins have been proposed to act as hepcidin inhibitors and "erythroid regulators": among them, erythropoietin (EPO) itself, soluble transferrin receptor-1 (TFR1), soluble hemojuvelin and hypoxia inducible factor-1 alpha (HIF1-α) [Camaschella C et al, 2015]. GDF15, a cytokine produced by mature erythroblasts, might play some role, as it may partially inhibit hepcidin transcription in primary hepatocytes. In addition, it was found in extremely high concentrations in the sera of beta-thalassemia patients [Tanno T et al, 2007], although this finding might simply reflect the release from ineffective erythropoiesis or from hypoxic cells.

The most recent candidate as regulator of hepcidin expression is the hormone erythroferrone (ERFE), a member of the C1q-tumor necrosis factor-related family of proteins (Figure 3). ERFE is an EPO-responsive gene. Mice injected with EPO rapidly (within 4 h) increased ERFE mRNA expression by erythroid precursors in the bone marrow and spleen through the stress erythropoiesis-related JAK2-STAT5 signaling pathway. ERFE plays an important role in ensuring iron supply during stress erythropoiesis *in vivo*. ERFE knockout mice failed to suppress hepcidin acutely in response to phlebotomy or EPO injections, indicating that ERFE is necessary for rapid hepcidin suppression in the setting of increased erythroid activity. β-thalassemia intermedia mice had dramatically increased ERFE mRNA levels in bone marrow and spleen, as would be expected for a condition characterized by high EPO levels and increased number of erythroid precursors [Kautz L et al, 2014; Kautz L et al, 2015].

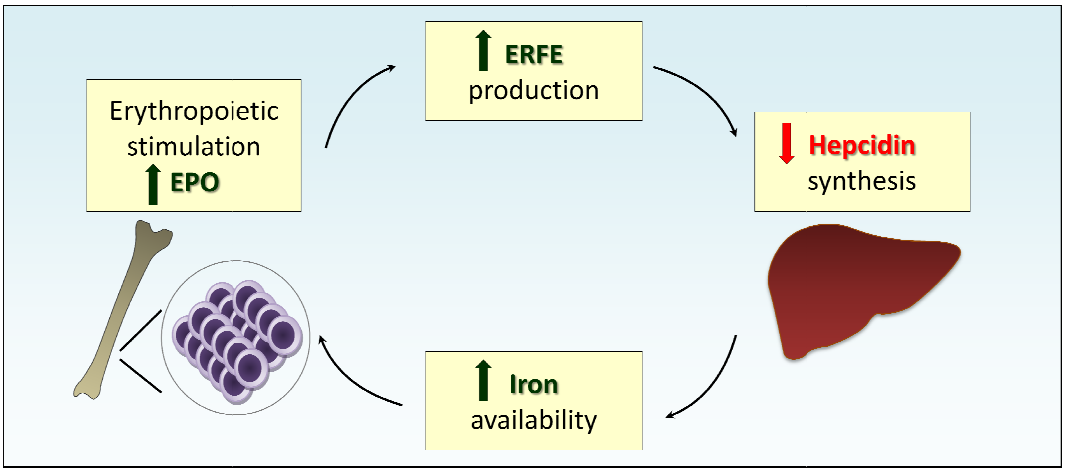

Figure 3: Proposed role of erythroferrone (ERFE).

During stress erythropoiesis, differentiating erythroblasts in the bone marrow and spleen rapidly increase ERFE production in an EPO- and JAK2/STAT5-dependent manner. ERFE is secreted into the circulation and acts on the liver via an unidentified pathway to repress hepcidin production. ERFE-mediated hepcidin suppression in turn increases iron availability for new red blood cell synthesis in the bone marrow.

1.3 Iron accumulation in beta-thalassemia carriers

In thalassemia major, iron overload is an unavoidable consequence of regular transfusions, which provide around 6 to 10 grams of iron per year, because the human body lacks a mechanism to excrete excess iron.

The iron loading in patients with non-transfusion-dependent thalassemia (NTDT) occurs more slowly than that in patients with thalassemia major, and is mainly due to increased intestinal absorption secondary to chronic anemia and ineffective erythropoiesis as a consequence of hepcidin suppression. Depending on the degree of the ineffective erythropoiesis, bone marrow expansion and peripheral hemolysis, the enhanced iron absorption accounts for the accumulation of about 2 to 5 grams of iron per year. Although the rate of iron loading is slower, patients with NTDT can eventually develop complications similar to those of patients with thalassemia major, including hepatic, endocrine and cardiac dysfunction, particularly as they advance in age [Origa R et al, 2007; Musallam KM et al, 2012].

A certain degree of IO is sometimes seen also in subjects with β-thalassemia trait. The pathogenesis is still unclear, but recently a population study in Sri

Lankan children has showed that βTT is characterized by mild hepcidin suppression due to increased erythropoietic activity [Jones E et al, 2015].

Other two recent studies have investigated the relationship between erythropoiesis and iron metabolism in β-thalassemia carriers in adult [Guimarães JS et al, 2015] and in pediatric age [Sulovska L et al, 2016], through an in-depth analysis of multiple selected parameters relating to both erythropoiesis (EPO, soluble transferrin receptor-sTfR and growth differentiation factor 15) and iron status (serum iron, ferritin, transferrin saturation and hepcidin). βTT carriers showed significant differences for all hematological parameters (hemoglobin concentration, RBC counts, MCV), while serum iron, ferritin and transferrin saturation levels were similar to those observed in healthy group. Hepcidin level was only mildly increased, without achieving statistical significance.

The Authors, in particular, analyzed the levels of sTfR (that reflects the entity of erythropoietic activity) and the hepcidin:ferritin ratio (which represents a measure of appropriate response of hepcidin to iron stores): in comparison with healthy controls, βTT subjects has a normal to low hepcidin:ferritin ratio (that suggests an inappropriate suppression of hepcidin synthesis) and concomitantly elevated sTfR (in accordance with an expansion of erythropoiesis). When they combine this parameters in the formula (hepcidin/ferritin)/sTfR (utilized to explore and quantify the opposing forces, i.e. iron availability and erythropoietic activity), the Authors observed that this ratio was reduced in β-thalassemia carrier group compared to healthy individuals and it distinguished thalassemia carriers from healthy controls. Notably, this finding confirmed a disordered interaction between iron metabolism and erythropoiesis in βTT subjects, also suggesting a possible greater susceptibility to iron accumulation compared with general population.

Although IO in βTT subjects is itself a quite rare condition, in individual patients numerous genetic (e.g. mutations in hemochromatosis genes) or acquired co-factors (e.g. alcohol abuse or non-alcoholic liver diseases) may further aggravated the relative hepcidin defect contributing to establish a progressive significant iron accumulation (Figure 4). In some cases iron overload results from

inappropriate long-term administration of oral or even parenteral iron, because of diagnostic errors in defining microcytic anemia.

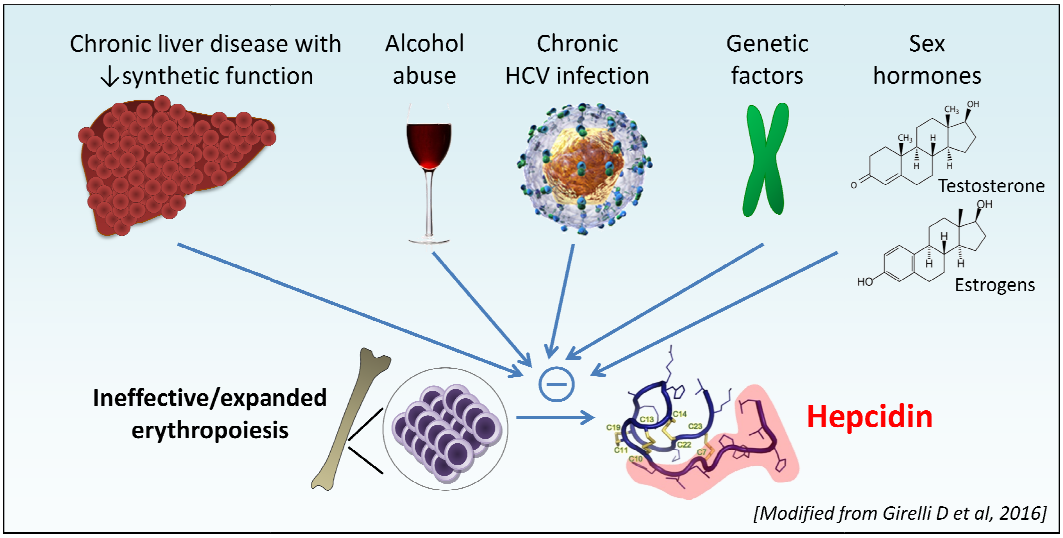

Figure 4: Genetic and acquired factors may aggravate hepcidin defect in βTT.

Multiple factors may aggravate hepcidin defect in βTT patients, such as chronic liver diseases [Tan TC et al, 2012], alcohol abuse [Dostalikova-Cimburova et al, 2014], hepatitis C virus (HCV) infection [Girelli D et al, 2009], hemochromatosis-related mutations [Girelli D et al, 2011; van Dijk BA et al, 2008; Papanikolaou G et al, 2005], and administration of the sex hormones testosterone [Bachman E et al, 2010; Guo W et al, 2013] and estrogens [Yang Q et al, 2012; Lehtihet M et al, 2016].

Concerning hereditary factors, their role is still not well defined. Variants on "hemochromatosis genes" (such as HFE, hemojuvelin, hepcidin, transferrin receptor 2 and ferroportin) could theoretically contribute to iron accumulation worsening the hepcidin defect. Although hereditary hemochromatosis (HH) is typically an autosomal recessive disorder and needs of a double mutation to develop, in β-thalassemia carriers is possible that even single mutations or polymorphisms may contribute to the iron accumulation. However, to date, a limited number of reports have specifically addressed this issue, with partially conflicting results: some suggested that iron overload might arise from the interaction of the beta-thalassemia trait with heterozygosity for hemochromatosis, some with homozygosity for hemochromatosis, and others that it was unrelated to hemochromatosis [Arruda VR et al, 2000; Piperno A et al, 2000; Melis MA et al,

2002; Martins R et al, 2004; Garewal G et al, 2005; Yamsri S et al, 2007; Madani HA et al, 2011; Nadkarni AH et al, 2016]. As shown in Table 3, these studies are markedly different each other and result difficult to compare.

Table 3: Principal studies evaluating the combined effects of βTT and HFE variants on iron status.

Reports	Aim of the study	Results/ Discussion
Piperno et al. 2000	To analyze phenotype, iron indices and HFE genotypes of 22 patients with the βTT who met the phenotypical criteria for HH (assessed by serum iron indices and liver iron concentration). To compare serum iron indices in relatives heterozygous for the C282Y mutation with and without the βTT.	βTT aggravates the clinical picture of C282Y homozygotes, favouring higher rates of iron accumulation and the development of severe iron-related complications. Association of the βTT with a single C282Y or H63D allele seems to not lead to iron overload.
Arruda et al. 2000	To describe modification of ferritin and transferrin saturation (TS) in one family comprising subjects C282Y mutation on HFE in homozygosis or heterozygosis, with and without βTT.	The propositus (female, 38 years old), homozygous for the C282Y mutation and heterozygous for β-thalassemia, presented a very high serum ferritin level, uncommon for patients at this age. Her mother, who carried βTT and C282Y mutation, presented a level of ferritin and TS slightly above the normal. On the contrary, her father, who does not present the βTT but is heterozygous for the C282Y mutation, did not have abnormal levels of ferritin or TS.
Melis et al. 2002	To evaluate the effect of the H63D mutation on the ferritin levels in 152 healthy males heterozygous for β-thalassemia (45 subjects heterozygous for H63D variants, 4 subjects homozygous for H63D).	Only the homozygous state for H63D is able to produce statistically significant higher levels of ferritin in βTT carriers.
Martins et al. 2004	To evaluate the effect of HFE mutations (C282Y, H63D, and S65C) on the iron status of βTT carriers (n=101) compared with controls (n=101).	H63D mutation, even when present in heterozygosity, may increased serum iron and TS in βTT carriers. The number of subjects carrying C282Y or S65C mutations was too low to conclude their effect on the iron status.
Garewal et al. 2005	To correlate C282Y and H63D mutations with the iron status in 215 βTT carriers.	All individuals were wild-type for the C282Y variant. No statistically significant difference in ferritin levels and TS was

		detected between the individuals of the wild-type and mutant for H63D.
Yamsri et al. 2007	To determine the prevalence of the H63D mutation in 370 Thai thalassemia carriers and 201 normal subjects and to evaluate its influence on ferritin level.	The H63D heterozygosity was identified in 5.5% of normal subjects and 7.3% of βTT carriers. The H63D heterozygosity has no significant effect on the serum ferritin in both groups.
Madani et al. 2011	To determine the prevalence of C282Y, H63D and S65C mutations of HFE gene in βTT and investigate their influence on TS, comparing them with individuals without thalassemias.	H63D, S65C and C282Y allele frequencies were 30.5%, 13.4% and 7.3% respectively in βTT and 10.0%, 2.5% and 0.0% respectively in the control group. 8 patients had TS >45% (7 males and 1 female). Of these, 6 were heterozygous for H63D, S65C and C282Y, 1 patient was heterozygous for H63D and S65C and 1 patient was homozygous for H63D.
Nadkarni et al. 2016	To look at the effect of HFE mutations on the iron status (ferritin level). A total of 100 βTT patients and 100 normal individuals were screened for the C282Y and H63D mutations on HFE.	βTT with H63D genotype showed higher ferritin levels as against wild type genotype. The study suggests that iron load in βTT tends to aggravated with the co-inheritance of the H63D mutation, even when present in heterozygosity.

1.4 Treatment of iron overload in beta-thalassemia carriers

Treatment of IO in βTT is problematic, since "standard" large-volume phlebotomies (400-450 ml) are not feasible in mildly anemic subjects. The use of the oral iron chelator deferasirox, although has been approved in the treatment of patients with non-transfusion dependent iron loading anemias, is not supported by extensive scientific evidence in patients with βTT and in some cases is burdened with serious complications such as acute renal failure.

Deferoxamine (DFO), available in clinical practice for nearly 50 years, remains therefore the iron chelator currently most used in βTT carriers with IO, but it is poorly applicable because of inconvenient parenteral administration and side effects. DFO is a large hexadentate iron chelator, with a low oral availability and a short half-life (approximately 20 min), that requires a parenteral administration, usually as a slow subcutaneous infusion via a portable pump for a period of 8-12 hours 5-7 nights per week. Average dosage is 20-40 mg/kg body weight for children and 30-50 mg/kg body weight for adults. The most frequent adverse effects of DFO are local reactions at the site of infusion, such as pain, swelling, induration, erythema, burning, pruritus, wheals and rash, occasionally accompanied by fever, chills and malaise. Other complications are sensorineural hypoacusia, ocular toxicity and infections by *Yersinia Enterocolitica*, and other pathogens like *Klebsiella Pneumoniae*. It is therefore important to monitor patients receiving DFO regularly with audiometric and ophthalmologic tests. However, because of the side effects and the inconvenient parenteral administration, a consistent proportion of patients is non-compliant, limiting the usefulness of this chelator, particularly in asymptomatic patients.

Sporadic case reports have suggested the use of phlebotomies in βTT patients with IO, but feasibility and efficacy of such approach has not been evaluated in patients' series. De Gobbi and colleagues, in particular, described the case of a young Italian female with juvenile hemochromatosis who was unable to tolerate frequent phlebotomy because of coexistent βTT. As an alternative to iron subcutaneous chelation, the patient was successfully iron-depleted by combining phlebotomies (350 ml) with recombinant human erythropoietin [De Gobbi M et al, 2000].

1.5 Aims of the study

This study has been designed to better characterize factors involved in the development of relevant IO in βTT patients, and to evaluate feasibility and efficacy of phlebotomies to remove IO in this condition. Given the potential risks associated with the use of erythropoietic-stimulating agents (ESAs) and the lack of specific guidelines, we have opted for a pioneering approach based on the use of "mini-phlebotomies" of 150-250 ml, without the addition of ESAs.

2. PATIENTS AND METHODS

2.1 Patients enrollment, blood sample collection and analysis

Until now, the study included 32 beta thalassemia carriers, referring to the Regional Referral Center of Iron Disorders of Azienda Ospedaliera Universitaria Integrata of Verona (Italy). The recruitment of subjects is still ongoing. The study was approved by the local ethical committee. All subjects released a written informed consent for hepcidin measurement and DNA analysis. The subjects were selected, since 1999, from outpatients who were referred to us for abnormalities of serum iron parameters.

Fasting blood samples were obtained in the early morning. Serum was obtained after centrifugation; if not analyzed immediately, samples were aliquoted and stored at -80°C for further analysis. Blood cell counts and erythrocyte indexes were determined with an automated cell counter. Other blood tests, serum iron, transferrin, and ferritin were determined by standard methods. For our study, the normal ranges we established for ferritin, serum iron and ST were 30–300 ng/ml, 55–160 μg/dl and 20–40%, respectively.

Hepcidin analysis was performed using an updated and validated Mass-Spectrometry (MS)-based assay, which is able to dissect the iron bioactive hepcidin-25 from other hepcidin isoforms [van der Vorm LN et al, 2016]. We used a liquid chromatography tandem mass spectrometry (LC-MS/MS) approach [Wolff F et al, 2013]. Briefly, hepcidin-25 synthetic standards (native and the isotopic labeled internal standard), as well as standards for hepcidin-20 and hepcidin-24 isoforms, were purchased from Peptide International (Lousiville, USA). Internal standard was added in all samples and calibration curve. Blank serum (deprived of hepcidin by using charcoal treatment) was used as reference. Samples were treated by solid phase extraction using Oasis hydrophilic-lipophilic balanced reversed-phase (HLB) cartridges (Waters, Italia). High Performance LC was performed using an X-Terra MS C18 2.5 Lm column (Waters, Italia), and detection was obtained using a Triple Quad LC MS/MS (Agilent Technologies). Results were evaluated according to previously obtained reference ranges (as reported in Table 4), for males and females at different ages [Traglia M et al,

2011]. The lower limit of detection of hepcidin with this method is 0.55 nM. In order to produce comparable results and to override the circadian rhythm of hepcidin [Ganz T et al, 2008; Kroot JJ et al, 2009] measurements were performed on samples obtained in all cases after an overnight fast.

Table 4: Normal values of hepcidin-25 (nM) according to sex and age.

Age class (years)	Males	Females
18-29	11.3 (1.3)*	4.4 (0.4)
30-39	11.8 (0.9)	4.7 (0.4)
40-49	11.4 (0.8)	4.7 (0.4)
50-59	10.8 (0.7)	11.1 (0.9)
60-69	11.8 (0.9)	11.6 (0.7)
70-79	11 (0.9)	10.2 (0.9)
≥80	10.6 (1.3)	8.6 (1.8)

**Reference values expressed as mean ± standard deviation*

The DNA samples were used for Next Generation Sequencing (NGS)-based test of the five gene involved in Hereditary Hemochromatosis (HFE, hemojuvelin [HJV], hepcidin [HAMP], transferrin receptor 2 [TfR2] and ferroportin [SLC40A1]) and of other 65 genes involved in iron homeostasis (e.g. BMP/SMAD1/5/8-signaling complex involved in hepcidin encoding gene expression) using IlluminaHiSeq 1000 platform, available at the Interdepartmental Functional Genomic Facility Centre of the University of Verona. Results were analyzed by the GoldenHelix™ method software [for details see Badar S et al, 2016].

Finally, serum ERFE concentration will be soon measured by the team of professor Nemeth in Los Angeles (the human assay for its measurements is currently under development).

2.2 Assessment of βTT carriers

The recognition of βTT carriers was based on hematological tests. The presence of a very low mean corpuscular volume (MCV) of red blood cells (RBC) with concomitant increased RBC number, with or without a mild anemia, suggested the diagnosis of βTT. All patients had a family history for microcytic anemia and, in some of them, we confirmed the presence of heterozygosis for a β-

gene mutation by a molecular test (PCR-based procedure) (genetic test for beta-thalassemia mutations is still in progress in other subjects).

2.3 Assessment of iron overload

Iron accumulation was firstly suggested by high ferritin levels (often higher than 1000 microgram per liter) and, in most cases, by high transferrin saturation and then confirmed by liver biopsy or magnetic resonance imaging (MRI).

Although raised levels of ferritin often indicate iron overload, they are not specific, as ferritin is an acute phase reactant and is also released from damaged hepatocytes; thus levels are elevated in inflammatory disorders, liver diseases, alcohol excess, or malignancy. Raised ferritin levels therefore require further investigation to determine if they truly represent iron overload. This can usually be confirmed through clinical assessment and measurement of serum transferrin saturation.

Moreover, serum ferritin levels seem to underestimate iron load in non-transfused thalassemia patients compared with transfusion-dependent patients. Some studies [Taher et al, 2009; Origa et al, 2007], in particular, found that serum ferritin was significantly lower in patients with thalassemia intermedia than in those with thalassemia major, despite the liver iron concentration being comparable. Lower serum ferritin in thalassemia intermedia reflects iron accumulation predominantly in hepatocytes rather than in macrophages, as observed by Perls' stain. This phenotype is likely the consequence of hepcidin deficiency. Hepcidin deficiency likely allows greater export of iron from macrophages, thus lowering macrophage cytoplasmic iron and suppressing secretion of soluble ferritin.

Although there are no studies that have specifically investigated the correlation between ferritin and iron accumulation in βTT carriers, it is conceivable that this last condition is more similar to that of TI than TM, being characterized by relatively suppressed hepcidin levels. It was therefore not excluded that ferritin tends to underestimate the actual iron deposits even in the βTT carriers.

Determination of liver iron concentration in a liver biopsy specimen shows a better correlation with total body iron accumulation and is considered the gold

standard for the evaluation of iron overload. Liver sections were stained with standard methods for histological evaluation and with Perls' stain for iron grading. However, liver biopsy is an invasive technique with the possibility (though low) of complications. Moreover, the presence of hepatic fibrosis or cirrhosis and heterogeneous liver iron distribution can lead to possible false negative results.

In recent years, nuclear magnetic resonance imaging (MRI) techniques for assessing iron loading in the liver (and heart) have been introduced. R2 and T2* parameters have been validated for liver iron concentration. In our patients, liver iron concentration (LIC) assessed by MRI was calculated by Gandon's algorithm (available on www.radio.univ-rennes1.fr) [Gandon Y et al, 2004]. In clinical practice, values less than 36 μmol Fe/g represent the physiological condition. Values less than 100 μmol Fe/g are indicative of mild iron overload while values greater than 200 μmol Fe/g suggest severe overload. The method, however, have some limitations: a) it saturates with very high iron overload and does not give a value of LIC higher than 350 μmol Fe/g [Alústiza Echeverría et al, 2012], b) has a tendency to overestimated iron overload [Castiella A et al, 2011] and c) is influenced by the presence of steatosis.

2.4 Therapeutic approach with "mini-phlebotomies" and calculation of iron removed

Traditional treatment by phlebotomy was unfeasible in our patients with mild anemia. As an alternative to iron chelation with DFO, in some selected patients we started a pioneering approach with "mini-phlebotomies" (150-250 ml). Frequency and amount of phlebotomies were established by the colleagues of the transfusion center and personalized based on the individual characteristics of each patient, in terms of hematological and clinical tolerability to the treatment.

In the presence of a normal value of hematocrit (around 45% for males and 43% for females), 1 ml of blood contains about 0.5 mg of iron. Calculating the mean blood hematocrit in patients with βTT, we hypothesized that is possible, although approximately, to estimate the amount of iron removed for each ml of blood taken during venesection in these mild anemic patients, according to the following proportion:

Iron removal in 1 ml blood (Ht 45%) = 0.5 mg Fe

normal Ht% : mean Ht% in βTT = 0.5 mg Fe: X mg Fe

Finally we calculated total iron removed multiplying the amount of iron estimated in each ml of blood and the total volume of blood removed at the end of venesection.

3. CRITICAL RESULTS

3.1. Clinical, biochemical, molecular data and iron status

Currently the study includes 32 subjects (25 males and 7 females), aged 23-80 years (mean age 59 years). The majority of subjects comes from Northern Italy. The mean values of hemoglobin were not uniform, in agreement with the known phenotypic variability of the β-thalassemic trait (Table 5).

Table 5: General characteristic of population sample and hematological data.

Patients	Sex	Age (years)	Origin	Mean Hb (g/dl)	β-gene mutation
1	M	54	Verona	12-13	n.a.
2	M	57	Verona	12-13	Codon 39 C>T
3	M	61	Rovigo	12-13	n.a.
4	M	40	Verona	12-13	n.a.
5	M	50	Verona	9.5-10.5	n.a.
6	M	59	Verona	13-14	n.a.
7	F	47	Verona	10-11	n.a.
8	M	52	Padova	11-12	n.a.
9	F	55	Verona	10-11	Codon 39 C>T
10	M	46	Verona	10.5-11.5	Codon 39 C>T
11	M	64	Verona	11.5-12.5	Codon 39 C>T
12	M	70	Verona	12-13	n.a.
13	F	64	Verona	10-11	Codon 39 C>T
14	M	80	Verona	14-15	n.a.
15	M	64	Verona	12.5-13.5	n.a.
16	M	64	Verona	11.5-12.5	Codon 39 C>T
17	M	65	Mantova	11-12	n.a.
18	M	48	Verona	11.5-12.5	IVS 2.745 C>G
19	M	75	Verona	11-12	Codon 39 C>T
20	M	60	Cagliari	12-13	n.a.
21	F	69	Napoli	10-11	n.a.
22	M	60	Treviso	13-14	IVS 1-nt110 G>A
23	F	46	Venezia	10.5-11.5	n.a.
24	M	56	Venezia	13-14	n.a.
25	M	23	Rovigo	13-14	n.a.
26	M	54	Verona	11-12	n.a.
27	M	65	Verona	12-13	n.a.
28	M	46	Trento	10-11	n.a.
29	M	75	Verona	12-13	n.a.
30	M	69	Verona	10.5-11.5	IVS 1.1 G>A
31	F	79	Verona	10-11	n.a.
32	F	67	Rovigo	11.5-12.5	n.a.

M: male; F: female; n.a.: not available

All subjects had high level of ferritin, detected in multiple determinations, and most of them had transferrin saturation higher than normal. No patient had clinical or biochemical signs of acute or chronic inflammation, while in approximately 40% there was a mild increase of transaminases due to coexistent chronic liver disease. 14 patients underwent to liver biopsy for diagnostic and/or prognostic aim. Liver iron concentration (LIC) was available in 20 subjects and was measured by quantitative magnetic resonance (MRI) as previously reported.

Combining these data (high ferritin levels, TS>40%, LIC-MRI>36 µmol/g and/or liver biopsy), we confirmed the presence of IO in 30/32 βTT carriers (Table 6). In 2 subjects (12 and 27, indicated by the arrows) we did not have enough elements to determine if the increase of ferritin levels was actually correlated to the presence of iron accumulation.

Table 6: Biochemical, imaging and histological data of population sample.

Patients	Ferritin (ng/ml)	TS (%)	AST/ALT (U/l)	LIC-MRI (µmol/g)	Liver Biopsy	Perls' staining	IO
1	944	54	n.a.	n.a.	S	Hep. (3+) + Kupf.	yes
2	962	55	↑	n.a.	S+C	Hep. (2+) + Kupf.	yes
3	1060	41	normal	150	FLH	Hep. (2+) + Kupf.	yes
4	993	43	↑	60	n.a.	n.a.	yes
5	2542	100	↑	>350	n.a.	n.a.	yes
6	691	19	normal	50	n.a.	n.a.	yes
7	735	28	normal	150	n.a.	n.a.	yes
8	1378	100	↑	n.a.	H+F	Hep. (4+)	yes
9	699	46	normal	330	S	Hep. (3+) + Kupf.	yes
10	947	100	↑	150	FLH+F	Hepatoc. (3+)	yes
11	1146	53	normal	270	S+C	Hep. (3+) + Kupf.	yes
12	*729*	*36*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*no*
13	848	65	normal	n.a.	n.a.	n.a.	yes
14	894	52	normal	n.a.	n.a.	n.a.	yes
15	644	40	normal	95	n.a.	n.a.	yes
16	620	52	normal	170	n.a.	n.a.	yes
17	1215	46	↑	170	n.a.	n.a.	yes
18	3087	98	↑	290	n.a.	n.a.	yes
19	454	66	normal	200	n.a.	n.a.	yes
20	1125	25	↑	65	FLH+F	Hep. (1+) + Kupf.	yes
21	840	77	↑	n.a.	C	Hepatoc.	yes
22	3650	87	normal	n.a.	F	Hep. + Kupf.	yes
23	994	88	↑	n.a.	F	Hep. (4+) + Kupf.	yes

24	3100	78	normal	n.a.	C	Hep. (3+)	yes
25	899	77	↑	65	FLH+F	Kupf.	yes
26	893	80	normal	55	n.a.	n.a.	yes
27	*450*	*26*	*normal*	*n.a.*	*n.a.*	*n.a.*	*no*
28	880	45	↑	270	H+F	Hep. (3+) + Kupf.	yes
29	1195	58	normal	250	n.a.	n.a.	yes
30	2642	91	↑	210	n.a.	n.a.	yes
31	841	61	↑	n.a.	n.a.	n.a.	yes
32	3015	70	normal	230	n.a.	n.a.	yes

TS: transferrin saturation. AST/ALT: aspartate and alanine transaminases. IO: iron overload. WT: wild type. S: steatosis. C: cirrhosis. FLH: fatty liver hepatitis. H: hepatitis (inflammatory damage). F: fibrosis. (°): after venesection.

As concern genetic factors potentially aggravating iron overload, we observed that four subjects carried a significant double mutation on HFE-gene (C282Y/H63D in patient 13 and C282Y/C282Y in patients 22, 23 and 24), while in most patients we found only the H63D variant (in homo- or heterozygosis) whose role in iron overload remains controversial.

Regarding acquired factors such as alcohol intake, considered that scientific communities in different countries agreed that "moderate" consumption of alcohol comprises about 15 g/day for women and 30 g/day for men (corresponding to 1–3 glasses per day of wine, containing 12–14% alcoholic fraction) [Biasi F et al, 2014], most of our subjects had a very high consumption of alcohol (about 28% declared a current or past alcohol consumption higher than 100 g per day and 12% around 60 g/day). One patient (subject 7) had been transfused with 30 U of RBCs in 1989 after a diagnosis of acute myeloid leukemia. Two patients (31 and 32) had been treated with prolonged parenteral iron therapy. Three subjects (18, 21 and 31) had a chronic hepatitis C infection.

Finally, about 31% of subjects showed at least two components of metabolic syndrome and 47% presented at least one component. The role of metabolic syndrome in the determinism of iron overload has been debating for a long time [Mendler MH et al, 1999; Riva A et al, 2008], however its presence represents an indisputable confounding factor in the interpretation of serum indices: hyperferritinemia with normal or mildly elevated transferrin saturation, in fact, is observed in approximately one-third of patients with non-alcoholic fatty liver disease (NAFLD) or metabolic syndrome and iron perturbations are frequently

observed in patients with obesity, insulin resistance or NAFLD [Datz et al, 2013; Datz et al, 2017]. This condition has been named the "dysmetabolic iron overload syndrome (DIOS)".

Table 7 summarizes main genetic and acquired factors potentially aggravating iron overload in our patients.

Table 7: Hereditary and acquired risk factors for iron accumulation.

Patients	HFE-mutation	Alcohol intake per day	Other acquired factors	Met Syn
1	H63D +/-	100 g	-	H + D
2	H63D +/-	100 g (until 1994)	-	-
3	WT	60 g	-	O
4	WT	60 g	-	DM+D+U+O
5	H63D +/-	100 g (until 1998)	-	D
6	WT	-	-	I+D+IFG
7	n.a.	-	RBC transfusion	-
8	WT	60 g	-	-
9	H63D +/-	-	-	-
10	WT	-	-	D + U
11	H63D +/+	150 g	-	-
13	C282Y/H63D	-	-	-
14	WT	-	-	U + D
15	WT	-	-	H + O
16	WT	150 g	-	O
17	WT	100 g (until 2013)	-	-
18	WT	> 100 g	HCV infection	-
19	WT	60 g	-	-
20	H63D +/-	-	-	H+O
21	H63D +/-	-	HCV infection	-
22	C282Y +/+	-	-	H
23	C282Y +/+	-	-	-
24	C282Y +/+	-	-	H+O
25	WT	-	-	IFG+U+O
26	H63D +/-	125 g (until 2013)	-	IFG+O
28	H63D +/-	-	-	-
29	WT	200 g (until 2015)	-	-
30	WT	-	-	-
31	WT	-	parenteral iron + HCV infection	H
32	WT	-	parenteral iron	-

Met Syn: Metabolic Syndrome factors. H: Hypertension. DM: Diabetes Mellitus. IFG: Impaired Fasting Glucose. D: Dyslipidemia. U: High Uric Acid. O: Overweight (body mass index >25 kg/m^2 and < 30 kg/m^2). RBC: red blood cells. HCV: hepatitis C virus.

The serum from 23/30 patients with IO was used for hepcidin determination by a Mass-Spectrometry (MS)-based assay (Table 8).

Table 8: Hepcidin level (nM) by mass spectrometry-based method.

Patients	Sex	Age	Hepcidin (nM)	Patients	Sex	Age	Hepcidin (nM)
2	M	57	7.20	**17**	M	65	14.83
4	M	40	11.80	**18**	M	48	6.10
5	M	50	33.00	**20**	M	60	13.98
6	M	59	22.60	**21**	F	69	1.87
7	F	47	23.90	**22**	M	60	< 0.55 (°)
8	M	52	4.70	**23**	F	46	3.58
9	F	55	6.90	**24**	M	56	< 0.55 (°)
10	M	46	6.40	**28**	M	46	12.37
11	M	64	11.80	**29**	M	75	28.00
13	F	64	14.90	**30**	M	69	22.80
15	M	64	15.40	**32**	F	67	20.20
16	M	64	13.00				

(°) after venesection

Figure 5 represent serum hepcidin levels in our βTT patients as compared to age- and sex-dependent hepcidin variations in a large sample of normal individuals [Traglia M et al, 2011].

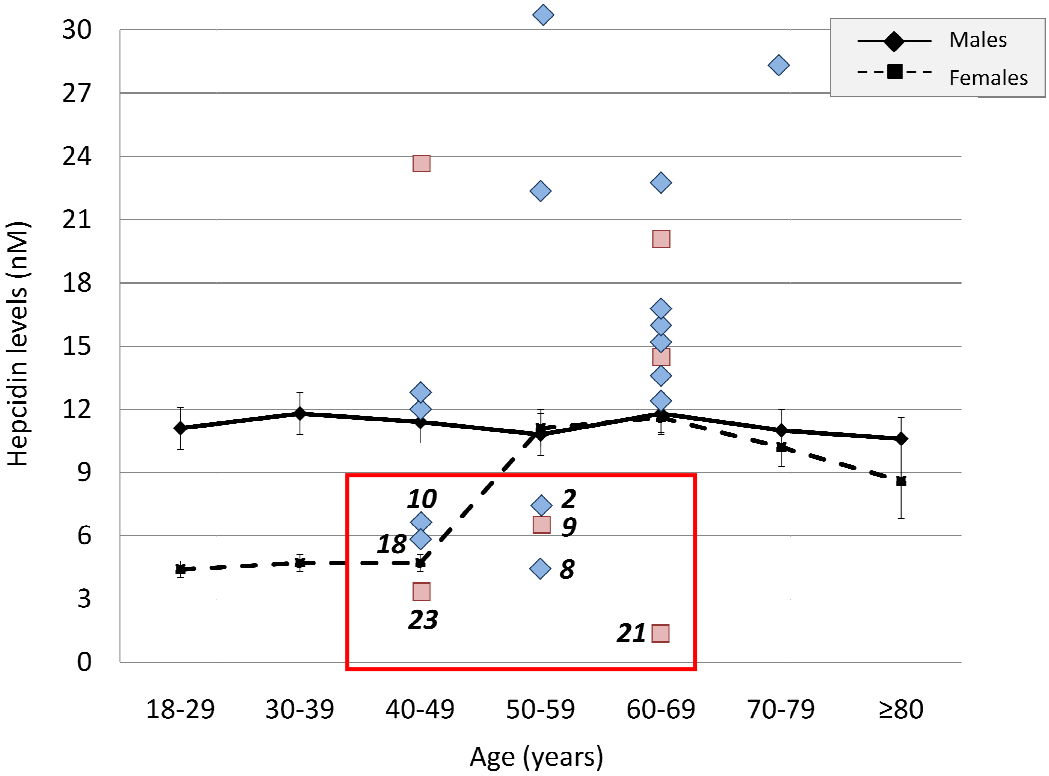

Figure 5: Serum hepcidin levels in our βTT patients with IO (diamond = males; square = females) as compared to age- and sex-dependent hepcidin variations in a large sample of normal individuals. In the red square are indicated the patients with hepcidin levels lower than normal.

In our population, 14/23 subjects had mean hepcidin levels higher than those observed in the general population, suggesting a certain degree of conservation of the hepcidin response to IO, while 7/23 subjects had mean hepcidin levels lower than normal. Hepcidin levels of patients 22 and 24 were not considered, because they were dosed after venesection.

Table 9 summarizes clinical, biochemical, genetic and histological characteristics of 7 subjects with hepcidin suppression. In particular, subject 23 had homozygous C282Y mutations on HFE gene, subject 2 had H63D variant and a previous very high alcohol intake, subject 21 had H63D variant and a chronic HCV infection, subject 18 had high alcohol intake and a HCV-related cirrhosis with a severe impairment of liver synthetic function.

Other patients (subjects 8, 9, 10) apparently did not exhibit significant aggravating factors for hepcidin defect but had clinical-biochemical or

histological characteristics indicating a possible non-HFE hereditary hemochromatosis.

Table 9: Clinical, biochemical, genetic and histological characteristics of 7 subjects with hepcidin suppression.

Patients	Sex	Ferritin (ng/ml)	TS (%)	HFE mutations	Acquired factors	LIC-MRI (µmol/g)	Perls' staining at liver biopsy
2	M	962	55	H63D +/-	daily alcohol intake of 100 g (until 1994)	n.a.	Hepatoc. (2+) + Kupffer
8	M	1378	100	WT	daily alcohol intake of 60 g	n.a.	Hepatoc. (4+)
9	F	699	46	H63D +/-	-	330	Hepatoc. (3+) + Kupffer
10	M	947	100	WT	-	150	Hepatoc. (3+)
18	M	3087	98	WT	daily alcohol intake of >100 g + HCV infection with liver cirrhosis	290	n.a.
21	F	840	77	H63D +/-	HCV infection	n.a.	Hepatoc.
23	F	994	88	C282Y +/+	-	n.a.	Hepatoc. (4+) + Kupffer

On the other hand, the hepcidin:ferritin ratio (Table 10), that reflects the response of hepcidin to iron stores concentrations, was lower in our βTT patients than in age- and sex-matched normal individuals, in agreement with a relative hepcidin defect in βTT.

Table 10: Hepcidin:ferritin ratio (pmol/g) in our population.

Patients	Sex	Age	Hep:ferr ratio	Patients	Sex	Age	Hep:ferr ratio
2	M	57	7.48	**17**	M	65	11.53
4	M	40	11.88	**18**	M	48	1.98
5	M	50	12.98	**20**	M	60	12.67
6	M	59	32.71	**21**	F	69	2.80
7	F	47	25.64	**22**	M	60	-
8	M	52	3.41	**23**	F	46	3.60
9	F	55	9.87	**24**	M	56	-
10	M	46	6.84	**28**	M	46	14.06
11	M	64	13.93	**29**	M	75	23.43
13	F	64	10.73	**30**	M	69	8.63
15	M	64	21.54	**32**	F	67	6.7
16	M	64	21				

Figure 6 represent hepcidin:ferritin ratio in our βTT patients as compared to normal individuals [Traglia M et al, 2011].

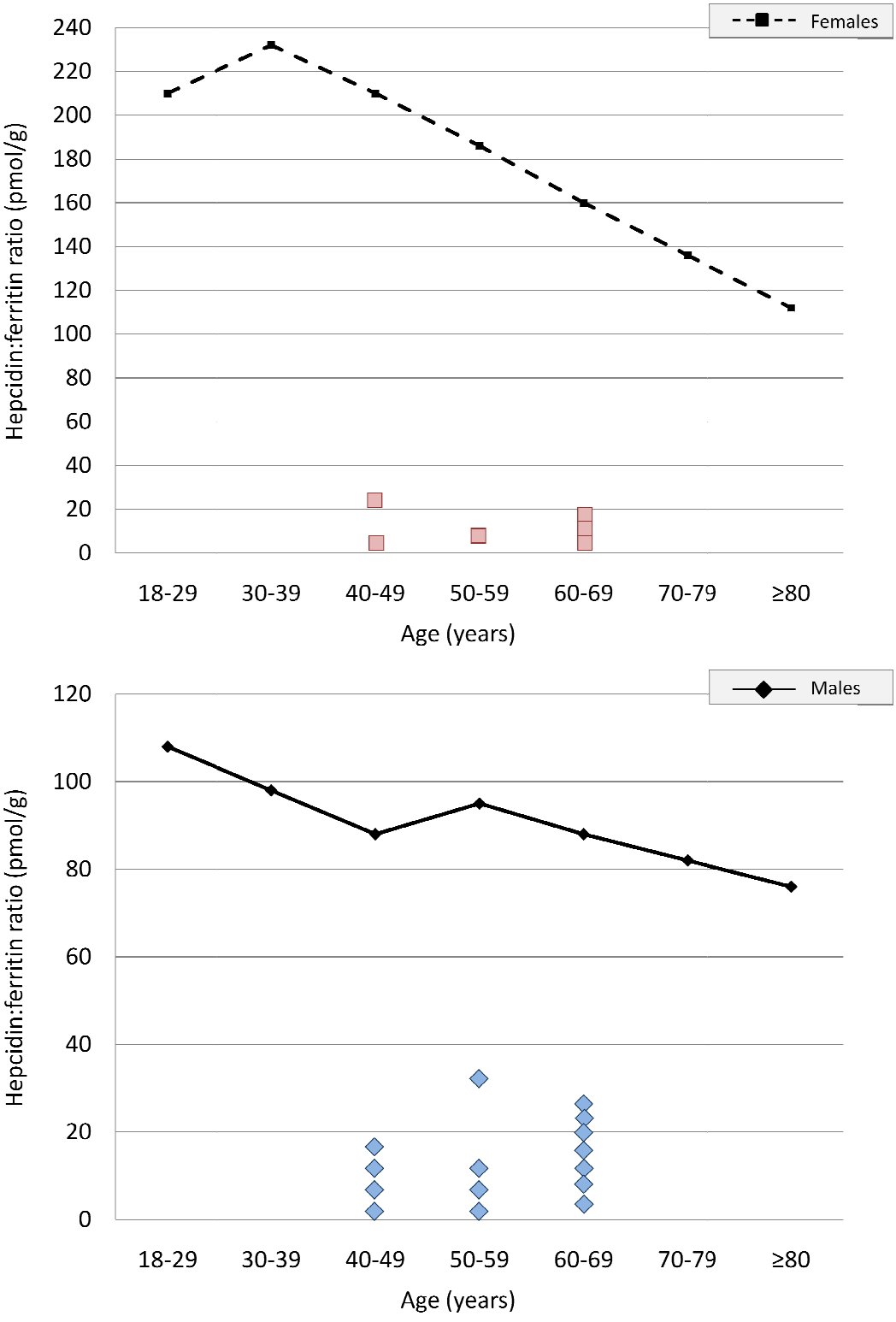

Figure 6: Hepcidin:ferritin ratio in our βTT patients with IO (diamond = males; square = females) as compared to age- and sex-dependent hepcidin variations in a large sample of normal individuals.

As concern Next Generation Sequencing analysis (up to now performed in 28 patients), it did not reveal potentially pathogenic variants out of the known H63D and C282Y in HFE, even in 5 subject with family history for liver disease or iron overload (Table 11).

Table 11: Family history for liver disease or hyperferritinemia.

Patient	Family History	βTT in relatives
1	cryptogenic cirrhosis (mother)	No
2	cirrhosis and hyperferritinemia (brother)	No
5	cirrhosis (brother and sister)	Yes (brother)
8	hyperferritinemia (father)	Yes
25	hyperferritinemia (father)	No

3.2 Patients treated with "mini-phlebotomies"

Fifteen patients started the treatment with "mini-phlebotomies". We treated one patient also with subcutaneous infusion of DFO, considered the degree of anemia (basal Hb 9-10 g/dl) and the severity of iron overload (ferritin 2542 µg/l, transferrin saturation 100% and LIC-MRI > 350 µmol/g). Phlebotomies were performed until iron depletion, that was achieved when ferritin was in the normal range (<300 ng/ml). Until now, eleven patients have reached the iron depletion.

None of the patients have experienced a worsening of anemia during the treatment. Only one subject temporarily discontinued the treatment because of a rapid decline of Hb level, due to a gastrointestinal bleeding (with subsequent endoscopic finding of "erosive gastritis Helicobacter Pylori related"). Table 12 summarizes number and frequency of phlebotomies and the estimated amount of iron removed (calculated as shown above).

Table 12: Characteristics of treatment with "mini-phlebotomies".

Patients	Mean Ht (%)	Phlebotomies (ml) and frequency	N° phlebotomies	Total blood removed (ml)	Initial ferritin (ng/ml)	Final ferritin (ng/ml)	Iron removed** (g)
2	n.d.	250 every 7 days	18	3500	962	146	n.d.
5	n.d.	~175 every 20 days*	22	n.d.	2542	n.d.	n.d.
7	34.2	150 every 7 days	54	8100	735	158	**3.2**
9	38.4	~150 every 15 days	42	7500	1378	261	**3.3**
10	33.5	100-150 every 15 days	39	5650	947	322	**2.1**
11	41.5	150-200 every 15 days	19	3000	1146	239	**1.4**
13	34.7	150 every 15-20 days	16	2400	848	143	**0.9**
15	38.6	150 every 15 days	16	2500	644	293	**1.1**
16	37.7	150-200 every15days	15	2950	620	302	**1.2**
19	34.6	200-250 every 30 days	18	3500	454	256	**1.3**
20	41.3	~250 every 10 days	16	4000	1125	104	**1.8**
21	n.d.	150 every 20 days	27 (in progress)	-	840	-	-
23	n.d.	150 every 15-20 days	30 (in progress)	-	994	-	-
28	n.d.	200 ml every 15-20 days	8 (in progress)	-	880	-	-
32	n.d.	250 ml every 10 days	15 (in progress)	-	3015	-	-

**plus DFO 20 mg/kg/die. **calculated in agreement with the degree of anemia (Ht)*

Although the estimation of iron removed is only approximate, it appears to be less than expected in relation to the initial values of ferritin and LIC-MRI and does not seem to have any significant correlation with all iron indices, included the transferrin saturation value (Table 13 and Figure 7, 8 and 9).

Table 13: Iron indices in βTT patients treated with "mini-phlebotomies" seem not to correlate with amount of iron removed.

Patients	Initial ferritin (ng/ml)	TS (%)	LIC-MRI (µmol/g)	Iron removed (g)
7	735	28	150	**3.1**
9	1378	46	330	**3.2**
10	947	100	150	**2.1**
11	1146	53	270	**1.4**
13	848	65	-	**0.9**
15	644	40	95	**1.1**
16	620	52	170	**1.2**
19	454	66	200	**1.3**
20	1125	25	65	**1.8**

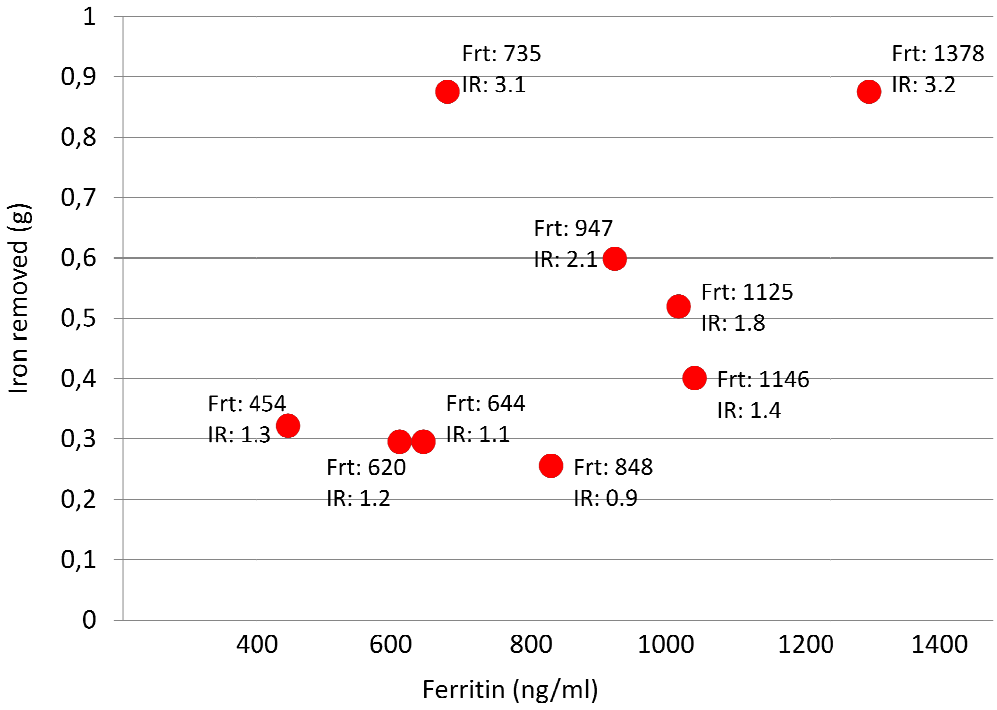

Figure 7: No correlation between **i**nitial ferritin value and IR in βTT patients treated with "mini-phlebotomies".

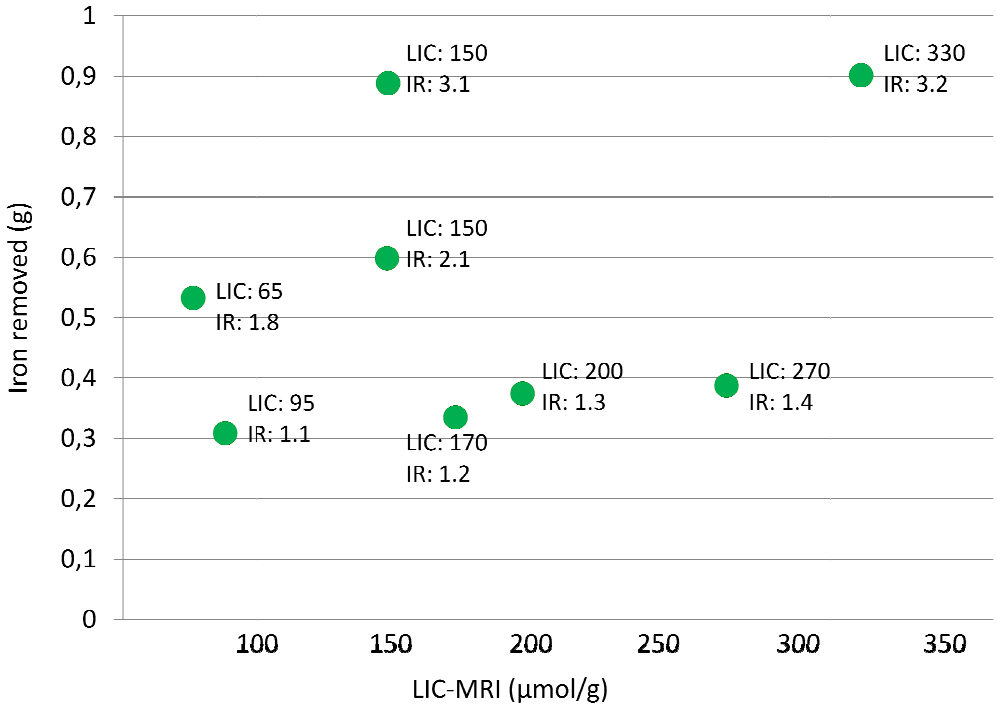

Figure 8: No correlation between liver iron concentration at MRI and IR in βTT patients treated with "mini-phlebotomies".

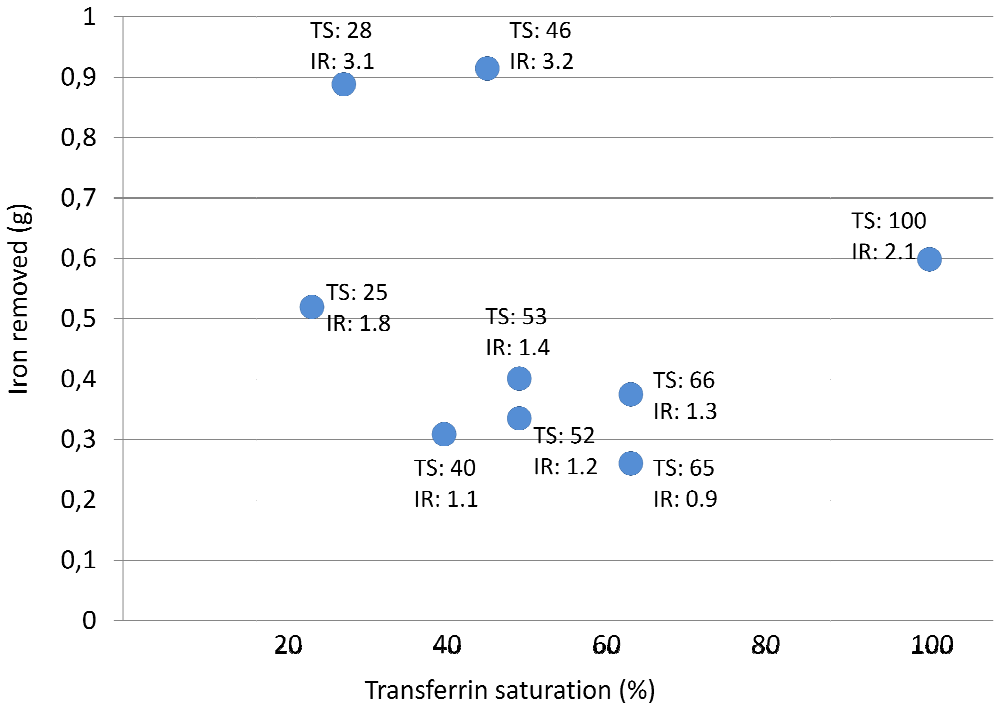

Figure 9: No correlation between transferrin saturation and IR in βTT patients treated with "mini-phlebotomies".

4. DISCUSSION

β-thalassemias are monogenic diseases characterized by the lack or reduction of the haemoglobin β-globin chain expression resulting in an increase of the α/β globin ratio. The excess free α chains aggregate and precipitate in erythroblasts, leading to damage of cell membranes and reactive oxygen species (ROS) generation, leading to ineffective erythropoiesis. Ineffective erythropoiesis is characterized by an expansion of immature erythroblasts associated with apoptosis of mature erythroblasts at the polychromatophilic stage, leading to a major reduction of red cell production. In addition, ineffective erythropoiesis of β-thalassemia is associated with iron overload. Patients with the most severe forms (β-thalassemia major) require chronic red blood cell transfusion for survival and iron chelation to prevent increased plasma iron and formation of non–transferrin-bound iron (NTBI) with its related organ damage.

Patients associated with a milder phenotype (β-thalassemia intermedia or non–transfusion-dependent thalassemia) may need only sporadic blood transfusions. However, because of ineffective erythropoiesis, these patients exhibit increased iron absorption and NTBI, leading to severe iron overload, its clinical manifestations, and eventually death. In addition, iron overload may further aggravate ineffective erythropoiesis by stimulating erythroblast ROS production, which increases the α/β-globin chains imbalance.

β-thalassemia trait with IO represents a relatively neglected and "orphan" condition, which can be observed in regions where βTT is prevalent along with certain multifactorial modifiers, particularly substantial alcohol intake and H63D variant on HFE gene.

In particular, the role of the H63D heterozygosis in IO determinism has been long debated, but recently an interesting study on its impact on the iron status and haemoglobin concentration during the developmental age, demonstrated that male H63D carriers presented higher blood iron, transferrin saturation and ferritin concentration than wild type probands, suggesting that changes in iron metabolism occur at young age in HFE heterozygotes, even in normal individuals [Barbara KH et al, 2016].

For the last decade, investigators have focused on understanding the mechanisms underlying iron overload in ineffective erythropoiesis, hypothesizing that correction of ineffective erythropoiesis would significantly reduce iron overload and improve anemia.

Hepcidin, a small peptide mainly produced by the liver, is absolutely required for the maintenance of systemic iron homeostasis in basal conditions. Hepcidin controls serum iron levels by binding to ferroportin (FPN), the only known iron exporter, and inducing its degradation. Low hepcidin stabilizes FPN at the cellular membrane, promoting dietary iron absorption in the duodenum, increasing the release of iron from macrophages following erythrophagocytosis, and enabling iron mobilization from hepatocytes. Likewise, hepcidin is suppressed in conditions associated with accelerated erythropoiesis (e.g. anemia due to bleeding, hemolysis, or iron deficiency) and ineffective erythropoiesis (e.g. β-thalassemia)

Although our βTT-IO patients display high levels of hepcidin, they show a very low hepcidin:ferritin ratio, suggesting that hepcidin response to iron is conserved in this subgroup of population but at a lower set point than in normal individuals. The inadequate hepcidin response demonstrated by the reduced hepcidin:ferritin ratio was in agreement with the findings of previous studies that found a reduced hepcidin:ferritin ratio even in subjects without overt iron accumulation [Jones E et al, 2015; Guimarães JS et al, 2015; Sulovska L et al, 2016].

In 2014, the Ganz laboratory has shown that ERFE, a member of the C1q-tumor necrosis factor–related family of proteins, is probably the major negative regulator of hepcidin in conditions of stress or ineffective erythropoiesis. ERFE is produced by erythroid precursors in the bone marrow on erythropoietin (EPO) stimulation and represses liver hepcidin production by a still unknown mechanism. These data have been confirmed in vitro and in mouse models, but still require confirmation in humans, so our population could provide extremely useful information about the mechanisms of reciprocal regulation between erythropoiesis and iron.

Preliminary results suggest “mini-phlebotomies” are effective in the removal of iron overload and overall well tolerated (none of the patients experienced a

worsening of anemia during the treatment), so they appear as a valuable approach for this peculiar category of mild anemic patients. However, in most patients, the calculated iron removed was less than expected on the basis of initial ferritin values and LIC. Concerning ferritin, this may be due to the presence of multiple confounders (active alcohol abuse, increased transaminases, DIOS). Magnetic resonance is known to overestimate the amount of iron accumulation, especially in patients with hepatic steatosis. The study is still ongoing and this point can probably be clarified by enrolling a larger number of βTT subjects without comorbidity factors.

In some subjects we observed a slightly increased of mean haemoglobin level after iron depletion, suggesting that iron overload may aggravate ineffective erythropoiesis. The mechanisms by which this occurs are not completely understood, but is known that iron overload inhibits burst-forming unit colony formation and erythroblast differentiation of both murine and human hematopoietic progenitors in vitro, and cells exposed to excess iron exhibit dysplastic changes with increased intracellular ROS and decreased BCL-2 (anti-apoptotic gene) expression [Taoka K et al, 2012].

To our knowledge this is the first study that investigated the role of "mini-phlebotomies" in the treatment of secondary iron overload in patients with mild anemia. This approach could be useful in other severely iron-loaded anemic patients.

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